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An Evaluation of Candidate Genes of Inflammation and Thrombosis in Relation to the Risk of Venous Thromboembolism: The Women's Genome Health Study

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Abstract

Background—While pathways associated with hemostasis and thrombosis are well-documented to impact upon venous thromboembolism (VTE), whether or not the inflammatory cascade also influences VTE risk is uncertain

Methods and Results—We evaluated 51 polymorphisms from 32 inflammation-related genes (and an additional 19 polymorphisms from 15 thrombosis-related genes) as potential determinants of venous thromboembolism (VTE) in a prospective cohort of 22,413 white women followed over a 10-year period. Hazard ratios for incident VTE according to the different genotypes were assessed by Cox proportional-hazards models. The false discovery rate (FDR) was used for correction for multiple testing with a 0.20 cut-point. During follow-up, 158 idiopathic and 180 secondary VTE events occurred. As anticipated, factor V Leiden (hazard ratio=3.22, 95%CI=1.92–5.40, $p<0.0001$, *FDR*=0.004), and the prothrombin mutation (hazard ratio=2.57, 95%CI=1.64–4.02, $p<0.0001$, *FDR*=0.004) were both strongly associated with incident idiopathic VTE, as was the rs6046 polymorphism in the factor VII gene (hazard ratio=0.54, 95%CI=0.35–0.86, $p=0.008$, *FDR*=0.12). With regard to polymorphism in the inflammatory genes, variation at rs1143634 in the interleukin-1 beta gene was associated with a reduced risk of idiopathic VTE (hazard ratio=0.59, 95%CI=0.44–0.80, $p=0.0007$, *FDR*=0.02) while variation at rs1800872 in the interleukin-10 gene was associated with increased risk (hazard ratio=1.42, 95%CI=1.12–1.80, $p=0.004$, *FDR*=0.07). By contrast, no significant associations were found for secondary VTE events.

Conclusion—In addition to previously reported polymorphisms associated with hemostasis and thrombosis, these prospective cohort data suggest that genetic variation in IL-1 beta and IL-10 genes may also influence the risk of idiopathic VTE.

Keywords

VTE; polymorphisms; candidate genes; risk factors

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Introduction

Genetic variation in multiple genes associated with hemostasis and thrombosis are well-documented to impact upon rates of future idiopathic venous thromboembolism (VTE) [1–7]. However, recent work has also suggested that inflammatory processes related to innate immunity may also play a causal role in VTE [8–10]. For example, systemic inflammation has been shown to be a potent prothrombotic stimulus, with effects including upregulation of procoagulant factors, downregulation of natural anticoagulants, inhibition of fibrinolytic activity, and modulation of platelet reactivity. Further, inflammatory mediators in the tumor necrosis factor and interleukin families, as well as complement factors have been shown to influence the pathogenesis of thromboembolism [1–4].

Despite these data, data evaluating common genetic variants within the inflammatory/immune cascade and VTE risk are sparse. To address this issue, we evaluated potential associations of 51 polymorphisms from 32 inflammation-related genes with risk of incident VTE in the Women's Genome Health Study (WGHS), a prospective cohort that included 22,413 initially healthy Caucasian women. As internal controls, we also evaluated 19 polymorphisms from 15 thrombosis-related genes, including factor V Leiden and the prothrombin mutation, two polymorphisms commonly ascertained in clinical practice.

Methods

Study participants

We studied DNA samples from a prospective cohort of apparently healthy women participating in the WGHS; a genetic sub-study of the Women's Health Study cohort, a recently completed randomized, double-blinded, placebo-controlled clinical trial of vitamin E and low-dose aspirin for the primary prevention of cardiovascular events and cancer among women [5,6]. Details of the WGHS study design have been described previously [6,7]. In brief, the WGHS cohort consist of nearly 25,000 American women free of cardiovascular disease or cancer at study entry who have been followed prospectively for future vascular events, including VTE. For the purposes of this analysis, we limited the study cohort to those women who self-reported ethnicity as white and who had no prior history of deep vein thrombosis nor pulmonary embolism (N=22,413). For any event of incident deep vein thrombosis (DVT) or pulmonary embolism (PE) reported during study follow-up, medical records, death certificates, and autopsy reports were obtained and reviewed by an endpoints committee to confirm or reject each diagnosis. The diagnosis of DVT required a positive result on venography or ultrasonography. The diagnosis of PE required a positive (CT) angiogram or a ventilation-perfusion scan that showed at least two segmental perfusion defects without ventilation defects.

Idiopathic VTE was defined as occurring in the absence of a known malignant condition (diagnosed either before or up to 3 months after the VTE) or trauma, surgery, or hospitalization (lasting ≥3 days) within 3 months before the VTE. Secondary VTE included events that occurred in patients with cancer or during or up to 3 months after trauma, surgery, or hospitalization (lasting ≥3 days) [8,9]. The proportions for PE alone, DVT alone, and PE-DVT were 14.7%, 60.2%, and 25.1%, respectively. Women who did not develop a VTE event during follow-up (neither idiopathic nor secondary) were considered as controls.

The study was approved by the Brigham and Women's Hospital Institutional Review Board for Human Subjects Research.

Selection of candidate gene polymorphisms

A total of 70 polymorphisms from 47 candidate genes were examined in each study participant; 51 polymorphisms were from 32 inflammation-related candidate genes, and 19 polymorphisms

were from 15 hemostatsis and thrombosis-related candidate genes. Polymorphisms were chosen based on prior evidence of potential functionality, validated allele frequency and heterozygosity, and sequence-proven allelic variation.

Genotype determination

Genotyping was performed using a previously described and validated linear-array assays for candidate markers of cardiovascular disease, immune response and inflammation (Roche Molecular Systems, Alameda, CA) 10›11. In brief, each DNA sample was amplified in multiplex polymerase chain reactions (PCRs) using biotinylated primers. Each PCR product pool was then hybridized to the corresponding panel of sequence-specific oligonucleotide probe that had been immobilized in a linear array on nylon membrane strips. The colorimetric detection method was based upon the use of streptavidin-horseradish peroxidase conjugate with hydrogen peroxide and 3,3′,5,5′-tetramethylbenzidine as substrates. Genotype assignment was performed using the proprietary Roche molecular systems strip scan image processing software. To confirm genotype assignment, scoring was carried out by two independent observers. Discordant results (<1% of all scoring) were resolved by a joint reading, and where necessary, a repeat genotyping.

Statistical analysis

We examined the association between each of the evaluated polymorphisms and risk of incident VTE in a multi-stage procedure. First, Hardy-Weinberg equilibrium was evaluated for each polymorphism using an exact test. The association of genotypes with VTE risk was evaluated using the Cox-proportional hazard regression analysis, assuming an additive model. All regression analyses were adjusted for age, body-mass index (BMI), treatment assignment, and use of hormone replacement therapy. Hazard ratios (HR) and the corresponding 95% confidence interval (CI) were calculated. As both genetic and environmental determinants of idiopathic VTE are known to differ from that of secondary VTE, statistical analysis was performed for each clinical endpoint separately.

For the purposes of epidemiological comparison, we used the false discovery rate (FDR) [12] to adjust for multiple hypothesis-testing. The FDR was applied to the adjusted models examining the additive effect of each gene variant on idiopathic and unprovoked VTE, separately, using the PROC MULTTEST of SAS, Version 9 (SAS Institute Inc., Cary, North Carolina). Unlike common procedures such as the Bonferroni correction, the FDR method does not control the experiment-wise error rate, but instead controls the expected proportion of false positives among all positive results over multiple studies. Although no universal FDR significance threshold has been defined, a cut-point of 0.20 has been suggested for candidate gene association studies 13, meaning that one should expect at most 20% of declared discoveries to be false.

The proportional hazards assumption was examined by including genotypic data by logarithm of time interaction into the model. All analyses were carried out using SAS/Genetics version 9 (SAS Institute Inc, Cary, NC). A two-tailed p-value of 0.05 was considered a statistically significant result.

All authors conceived and designed the study project. RYLZ conducted the experiments. SC and LS contributed reagents/materials. RYLZ, RJG and PMR discussed and analyzed the data. RYLZ prepared the manuscript draft. All authors finalized and approved the paper. The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results

Baseline characteristics of the study participants are shown in Table 1. The 70 genetic variants from 47 genes examined in the present investigation are presented in the online Supplementary Table 1; all in Hardy-Weinberg equilibrium after correction for multiple testing.

The observed allele frequencies of the remaining genetic variants tested are comparable to those reported on the National Center for Biotechnology Information SNP database for Caucasian/white population (http://www.ncbi.nlm.nih.gov/sites/entrez?db=snp). Furthermore, at the 0.05-alpha level, the polymorphisms tested were in proportional hazards assumption.

For ease of presentation, Table 2 presents those gene variants that were found to have a nominal p-value of less than 0.10 for the Cox regression analysis, in an additive model, for idiopathic, and secondary VTE, respectively. Online Supplementary Table 2 presents the nominal association results for all the gene variants tested.

As anticipated, both the well-recognized prothrombin mutation (*rs1799963*: HR=3.2, 95% CI=1.9–5.4, $p<0.0001$, $q=0.0035$) and factor V Leiden (*rs6025*: HR=2.6, 95%CI=1.6–4.0, $p<0.0001$, $q=0.0035$) were significantly associated with incident idiopathic VTE at low FDR. Among the remaining variants ascertained in the hemostatic and thrombotic genes, significant association with idiopathic VTE was also observed for factor VII *rs6046* (HR=0.55, 95% CI=0.35–0.86, $p=0.0084$, $q=0.12$; Table 2).

With regard to the inflammatory genes, two novel associations were found, a protective effect for polymorphism at *rs1143634* in the interleukin-1 beta gene (HR=0.59, 95%CI=0.44–0.80, $p=0.0007$, $q=0.016$), and a net hazard for rs1800872 in the interleukin-10 gene (HR=1.42, 95% CI=1.12–1.80, $p=0.0038$, $q=0.07$) (Table 2). In addition to these statistically robust findings, nominal associations with idiopathic VTE were also observed for factor VII *rs5742910* (HR=0.60, 95%CI=0.40–0.92, $p=0.018$, $q=0.20$) and for interleukin-6 *rs1800796* (HR=1.60, 95%CI=1.07–2.38, $p=0.022$, $q=0.22$) (Table 2).

We found no evidence of association for any of the polymorphisms tested with secondary VTE risk (Table 2).

Discussion

In this prospective study, in addition to the well-recognized prothrombin mutation and factor V Leiden, we found variation in two inflammatory genes (interleukin-1 beta *rs1143634* and interleukin-10 *rs1800872*) to significantly associate with risk of incident idiopathic VTE. In addition, corroborating our own prior work in men [11], we also found significant association between factor VII *rs6046* and idiopathic VTE; of interest, the magnitude and direction of effect for *rs6046* in the current data for women is almost identical to that previously reported in men. However, we found no evidence for an association of any gene evaluated with secondary VTE risk.

With regard to our novel findings, the protein encoded by the interleukin-1 beta gene is a member of the interleukin 1 cytokine family. This cytokine family is produced by activated macrophages as a proprotein, an important mediator of the inflammatory response as well as a procoagulant factor, and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis, and tissue-factor expression on endothelial cells and monocytes [14–16]. As noted previously [15–17], IL-1 has been shown to stimulate procoagulant activity and leukocyte adhesion in human endothelial cells cultured from both umbilical veins and adult saphenous veins but not in other cultured cell types. Similar actions

of IL-1 on vascular endothelium in vivo may contribute to the development of intravascular coagulation and enhanced leukocyte-vessel wall adhesion at sites of inflammation [15–17].

As for interleukin-10, the protein encoded by this gene is a cytokine produced primarily by monocytes and to a lesser extent by lymphocytes. These inflammatory proteins participate in the underlying pathophysiology of venous thrombosis via the induction of pro-coagulation, and inhibition of anticoagulation [4,18,19]. A study by Downing *et al*. showed that IL-10 was elevated in the vein wall during venous thrombosis. Neutralization of IL-10 increased inflammation, while supplementation with rIL-10 demonstrated a dose- and time-dependent decrease in inflammation. These data demonstrate an anti-inflammatory property of IL-10 in the regulation of thrombus-associated inflammation and thrombosis, and suggest that IL-10 could be used as a therapeutic agent in the treatment of venous thrombosis [2]. Furthermore, a recent study by Reitsman and Rosendaal (Leiden Thrombophilia Study) has shown that levels of various cytokines, including IL-1 beta and IL-10, were risk determinants for venous thrombosis [4]

In a study by Pieroni *et al*., evaluating the variation at TNF-alpha *308G>A* (*rs1800629*), LT-alpha *252A>G* (*rs909253*), IL-6 *174G>C* (*rs1800769*), IL-1ra 86bp VNTR, IL-10 *1082G>A* (*rs1800896*), and CD-31 *125C>G* (a.k.a. *PECAM1 rs668*) polymorphisms, no evidence of altered VTE risk was observed although co-inheritance of LT-alpha *252A>G* and IL-10 *1082G>A* was associated with a two-fold increase in thrombotic risk [20]. Other studies examining several inflammatory markers, including C-reactive protein and interleukin 6, showed little evidence of an association with VTE risk [21–24]. Taken together, our present and previous investigations suggest that the release of cytokines may be important in mediating the activation of both the coagulation and the fibrinolytic mechanisms in thrombosis.

In a recent study by Smith *et al*. [13], examining the association of common variants in 24 clotting-related candidate genes with risk of incident venous thrombosis in 2029 postmenopausal women, only the tissue factor pathway inhibitor gene demonstrated global association (q=0.13). In their single-marker analysis, five gene variants were found with q-value less than 0.20 (factor V *rs4524*, factor XI *rs2289252*, and protein C *rs1799810*, *rs2069915*, and *rs5937*). More recently, a report by Bezemer *et al*. [25] evaluating over 19,000 gene-centric variants with risk of deep vein thrombosis in three case-control sample series and using a q-value of 0.20 as cut-point, three gene variants (*CYP4V2 rs13146272*, *SERPINC1 rs2227589*, and *GP6 rs1613662*) were identified with q-values less than 0.10. However, as our gene-panel was initiated prior to these reports, and did not include these gene variants, direct comparisons could not be made.

The candidate gene approach relies on prior knowledge of biological pathways and its associations with the phenotype of interest. In recent years, genome-wide association studies of common, complex diseases have become available, and have provided insights in the underlying pathophysiological mechanisms of several common disorders. Unfortunately, to date, no large genome-wide association investigations have been conducted in relation to VTE, thus, highlighting the need for large-scale, prospective studies in this important clinical condition. In this context, in addition to the candidate gene set described here, the Women's Genome Health Study project will eventually include full genome-wide scan data (estimated completion March 2009); thus, more detailed results regarding other potential genetic predispositions to VTE are expected in future analyses [7].

Strengths of the present study are the overall sample size, the biological relevance of the polymorphisms considered, the prospective design and the complete long-term follow-up. We also chose, on an a priori basis, to adjust for multiple comparisons, and to present all our data simultaneously rather than focusing on any one specific finding. Nonetheless, some potential

limitations of our study require discussion. First, this study included only white female health professionals, and our findings may not be generalizable to other populations, and with different socioeconomic background. Furthermore, participants used in the present study were drawn from a clinical trial population (WHS), which is inherently different from a population-based study. Of note, while we observed only modest, non-significant associations of the factor V Leiden and prothrombin mutation with provoked/secondary VTE risk, confidence intervals for these estimated associations were wide and consistent with those observed in many previous studies [26–31]. Discrepancies among reports could be due to differences in study design, the definition of VTE events in varying settings, methods of surveillance for events, and/or chance. In the present study, the increased hazards of provoked events associated with each of the factor V Leiden and prothrombin mutation were not significantly different from the observed hazard ratios for idiopathic events (p=0.13 for factor V Leiden hazard ratios, provoked versus idiopathic events; p=0.10 for the prothrombin mutation hazard ratios, provoked versus idiopathic evenets) [32]. Thus, further prospective investigation of the factor V Leiden and prothrombin mutation in provoked/secondary VTE is warranted.

It is also possible that one or more of the observed associations is the result of linkage disequilibrium with a yet-to-be-identified nearby susceptibility locus(i) or gene(s). As previously described, the polymorphisms tested in our study were solely selected based on prior evidence of potential functionality, validated allele frequency and heterozygosity, and sequence-proven allelic variation, and thus linkage disequilibrium (LD)/haplotype structure was not considered. Hence, further studies using LD/haplotype-SNP tagging information from public genome databases such as HapMap, SeattleSNPs, are warranted; In this regard, the HapMap linkage disequilibrium (r-squared) plots for the ±10kb region of *IL1B rs1143634*, and *IL10 rs1800872* are presented in Supplementary Figures 1 and 2, respectively. More importantly, replication of our present findings in different populations is required, despite the low false discovery rates observed and the high level of significance found in our regression analyses.

In our study, we had the ability to detect, based on the present sample sizes, assuming 80% power, at an alpha of 0.05, a hazards ratio of greater than 1.42 (idiopathic VTE), and 1.40 (secondary VTE) if the minor allele frequency is 0.50, and of greater than 2.62 (idiopathic VTE), and 2.58 (secondary VTE) if the minor allele frequency is 0.01 assuming a univariable-additive model. Thus, we cannot rule out a modest risk of VTE associated with the polymorphisms tested. Hence, based on the observed hazards ratios and the corresponding confidence intervals, polymorphisms that are potentially false negatives after our multiple-testing correction warrant further investigation; as an example, interleukin-6 *rs1800796* has been implicated in various vascular conditions including venous thrombosis [4,33].

In conclusion, this prospective investigation among initially healthy white US women indicates that genetic variations in factor VII, interleukin-1 beta, and interleukin-10 may play a critical role in the pathogenesis of incident idiopathic venous thromboembolism. Additional large-scale studies using comprehensive genome-wide analysis are needed to further elucidate the pathogenesis of venous thromboembolism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Table 1

Baseline characteristics of white female study participants

Characteristic	N=22,413
Age	54.7±7.1
Body-mass index, kg/m^2	25.8±4.9
History of diabetes (%)	2.5
History of hyperlipidemia ≥ 240 mg/dL (%)	29.6
History of hypertension ≥ 140/90 mmHg (%)	24.5
Smoking (%)	
Current	11.6
Former	37.0
Never	51.4
Hormone replacement therapy use (%)	43.8
Aspirin use (%)	50.0
Beta-carotene use (%)	49.8
Vitamin-E use (%)	50.0

Data are mean±standard deviation or percentages

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Table 2

Multivariable Cox regression analysis of gene variants with a nominal p-value of <0.10*

		Idiopathic/unprovoked VTE (N=158 events)		Secondary/provoked VTE (N=180 events)	
Variant	**Minor allele**	**HR, 95%CI, *p***	**FDR**	**HR, 95%CI, *p***	**FDR**
NOS3 rs1800779	*G*	--	--	1.25, 1.02–1.55, 0.0354	0.7717
NOS3 rs3918226	*T*	--	--	1.52, 1.10–2.10, 0.0102	0.7140
F2 rs1799963	*A*	3.22, 1.92–5.40, <0.0001	**0.0035**	1.51, 0.73–3.16, 0.2684	0.9938
F5 rs6025	*Gln*	2.57, 1.64–4.02, <0.0001	**0.0035**	1.47, 0.85–2.56, 0.1694	0.9938
F7 rs5742910	*Insertion*	0.60, 0.40–0.92, 0.0176	0.2053	--	--
F7 rs6046	*Gln*	0.54, 0.35–0.86, 0.0084	**0.1176**	--	--
SELE rs5355	*Phe*	--	--	0.53, 0.26–1.06, 0.0730	0.9190
TNF rs1800629	*A*	--	--	1.32, 1.03–1.71, 0.0303	0.7717
ICAM1 rs5491	*Met*	--	--	2.71, 0.86–8.49, 0.0870	0.9190
CCR2 rs1799864	*Ile*	1.42, 1.02–1.99, 0.0406	0.3552	--	--
CCR5 rs1799987	*A*	--	--	1.24, 1.01–1.53, 0.0441	0.7717
IL1A rs1800587	*C*	0.79, 0.62–1.02, 0.0707	0.4949	--	--
IL1B rs1143634	*T*	0.59, 0.44–0.80, 0.0007	**0.0163**	--	--
IL6 rs1800796	*C*	1.60, 1.07–2.38, 0.0221	0.2210	--	--
IL10 rs1800872	*A*	1.42, 1.12–1.80, 0.0038	**0.0665**	--	--
NOS2A rs1137933	*T*	0.76, 0.57–1.01, 0.0561	0.4363	--	--
GC rs4588	*Lys*	--	--	0.81, 0.64–1.04, 0.0919	0.9190

HR, hazard ratio; CI, confidence interval; FDR, false discovery rate.

*Adjusted for age, body-mass index, randomized treatment assignments (aspirin, beta-carotene, and vitamin E), and hormone replacement therapy use. *F2 rs1799963* (prothrombin mutation) and *F5 rs6025* (factor V Leiden) are presented for both strata.